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REGULATORY EFFECTS OF AMMONIA ON CARBON METABOLISM IN *CHLORELLA PYRENOIDOSA* DURING PHOTOSYNTHESIS AND RESPIRATION

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SUMMARY

Addition of ammonia to *Chlorella pyrenoidosa*, respiring in the dark following a period of photosynthesis, causes a stimulation of the flow of carbon into the synthesis of amino acids similar to that observed upon addition of ammonia during photosynthesis. In both cases, this stimulation is due not only to the increased availability of NH_4^+ for reductive amination of α-ketoglutarate to glutamate but is also due to stimulation of the rate of conversion of phosphoenolpyruvate to pyruvate. Addition of NH_4^+ in the dark causes a large increase in the formation of 6-phosphogluconate, beyond the increase in 6-phosphogluconate already seen when the light is turned off. When the light is turned off, the level of starch begins to decrease, and the rate of this decrease is not changed by the subsequent addition of ammonia. In contrast, the level of sucrose becomes nearly constant when the light is turned off, but begins immediately to decline when ammonia is added. As observed before, the level of ATP drops temporarily when the light is turned off and then rises to a steady state similar to that seen in the light. Upon the addition of ammonia, a similar transient drop and re-establishment in the level of ATP is seen.

These and other reported results are discussed with respect to sites and mechanisms of light-dark metabolic regulation leading to increased flow of carbon from carbohydrate reserves into mitochondrial metabolism in the dark, and the sites and mechanisms by which ammonia affects the rate of this flow.

INTRODUCTION

Previous kinetic studies of ^{14}C-labelled compounds formed in *Chlorella pyrenoidosa*, photosynthesizing with $^{14}CO_2$ and during subsequent dark and light periods, revealed regulatory mechanisms which control the flow of carbon during photosynthesis and respiration, and regulate the flow of carbon from the photosynthetic carbon reduction cycle to biosynthetic metabolism[1-3]. These and other studies (recently reviewed[4]) indicated that within the chloroplasts, principal control points are the carboxylation of ribulose-1,5-diphosphate, and the hydrolyses of fructose-

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Abbreviation: PEP, phosphoenolpyruvate.

1,6-diphosphate and sedoheptulose-1,7-diphosphate (activated in the light) and the oxidation of glucose-6-phosphate to 6-phosphogluconate (activated in the dark). These controls permit exclusive operation of the reductive pentose phosphate cycle in the light and the oxidative pentose phosphate cycle in the dark.

Comparison of flow rates and pool sizes in synchronously-grown *Chlorella pyrenoidosa*, at various stages in the cell life cycle, suggested that changes in the activities of the enzymes mediating these controlled reactions may play a role in the distribution of reduced carbon from the reductive pentose phosphate cycle to biosynthetic metabolism[5]. These changes were correlated with changes in the rates of flow of carbon into amino acids and acids of the tricarboxylic acid cycle (low in small cells which had recently divided in the dark and high in rapidly growing cells) and rates of flow of carbon into sucrose (high in the small cells and low in the rapidly growing cells)[5]. The differences in rates of flow of carbon into amino acids were further correlated with the apparent intracellular NH_4^+ level. Thus, rapidly growing cells showed a high rate of amino acid synthesis when supplied with nitrogen in the form of NO_3^-, NO_2^-, or NH_4^+. Rapid amino acid synthesis in the small cells, which are unable to reduce NO_3^-, or NO_2^-, was seen only upon the addition of 1 mM NH_4^+ to the cell suspension medium[6].

Examination of the changes in pool sizes of intermediate compounds when 1 mM NH_4^+ was added to an unsynchronized cell suspension of *Chlorella pyrenoidosa* photosynthesizing with $^{14}CO_2$ but with no added nitrogen source in the medium, demonstrated that the conversion of phosphoenolpyruvate to pyruvate is an important control point in the flow of carbon from the reductive pentose phosphate cycle to amino acid biosynthesis[7]. Upon this addition, the level of 3-phosphoglycerate and phosphoenolpyruvate dropped suddenly while the level of pyruvate increased and the rate of formation of alanine increased, and the flow of ^{14}C into lipids and into citrate, malate, glutamate, aspartate and glutamine increased[7].

Similar effects have now been observed upon the addition of NH_4^+ to algae respiring in the dark after a previous period of photosynthesis with $^{14}CO_2$. However, there are some differences which provide additional information about the nature of light–dark regulation.

EXPERIMENTAL

Chlorella pyrenoidosa was cultured aseptically using the turbidostat[8] at 25° with a stream of air *plus* 4 % CO_2 (v/v). Cell density was kept constant at 0.5 % by adding culture medium (modified Myers medium)[5] automatically.

The cells were harvested, washed once with 0.1 mM KH_2PO_4 (pH 6.0) and resuspended (1 %) in this medium. 60 ml of the suspension and about 5 mC of $^{32}P_i$ were poured into the steady-state apparatus. The pH was adjusted to 6.0 with 0.1 M NaOH. After 30 min of photosynthesis at 20° with 1.5 % CO_2, $^{14}CO_2$ was added from a small loop to the closed system. Samples (approx. 1 ml) were taken into 4 ml of methanol at intervals, as indicated in RESULTS.

At 25 min after the introduction of $^{14}CO_2$ into the system, the light was turned off. At 12 min from turning off the light, 1 ml of 0.04 M NH_4Cl was injected into the suspension and the sampling was continued for another 13 min. The pH did not change appreciably during the experimental run, and adjustment was unnecessary.

The partial pressure of CO_2 at the start of the experiment was 1.7 % (specific activity, 12.4 μC/μM) and it was 1.1 % at the end of the light period. The detailed method of the steady-state experiment has been described elsewhere[3,9].

Three 500 μl portions of each sample were analyzed by two-dimensional paper chromatography, first with phenol–acetic acid–water and second with butanol–propionic acid-water[10]. One of the chromatograms was developed 24 h in each direction for analysis of the secondary products of photosynthesis. The second chromatogram was developed 48 h in each direction in order to separate the intermediates of the photosynthetic carbon reduction cycle and other phosphorylated compounds. The third chromatogram was developed 72 h in each direction with 0.1 μmole each of authentic UTP, UDP, UDPG, ATP and ADP added to the origin as markers.

A 200-μl portion of the sample was defatted with methanol, dried, hydrolyzed for 1 h at 100° and an aliquot (corresponding to 20 μl of the original) was analyzed by two-dimensional chromatography (each direction for 24 h) for starch–glucose analysis[5].

Location of the labeled metabolites was detected by radioautograms and the content of ^{14}C and ^{32}P was determined semiautomatically, as has been described in detail[3].

For analysis of pyruvate and α-ketoglutarate, a 2-ml portion of the sample with added authentic ketoacids was coupled with 2,4-dinitrophenyl-hydrazine, extracted and chromatographed using *n*-butanol–ethanol–0.5 M NH_4^+ (7:1:2, by vol.) in the dark, as described in previous experiments[7]. However, after cutting the spot of pyruvate hydrazone, further 24 h chromatography with the same solvent was necessary for α-ketoglutarate hydrazone to separate completely from the radioisotope contaminant near the origin.

RESULTS

When the light was turned off, the levels of phosphoglycerate (Fig. 1), phosphoenolpyruvate (Fig. 2) and pyruvate (Fig. 3) in the algae which had been photosynthesizing without added nitrogen in the medium each rose rapidly, peaked, and dropped back to levels comparable to the steady-state light levels (pyruvate and

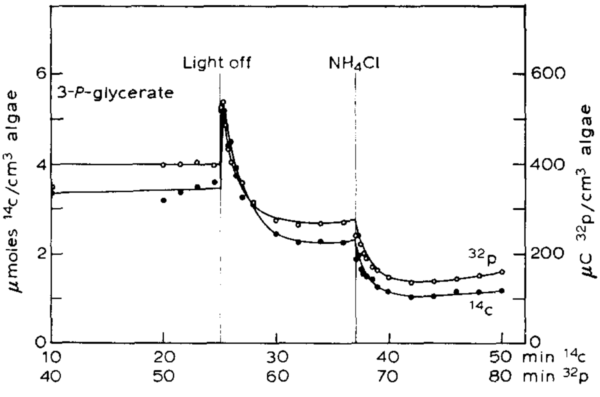

Fig. 1. 3-Phosphoglycerate. ○, ^{32}P label: ●, ^{14}C label.

Figs. 1–15. Levels of ^{32}P-labelled and ^{14}C-labelled metabolites in *Chlorella pyrenoidosa* during photosynthesis, respiration, and respiration with added NH_4^+.

phosphoenolpyruvate) or lower (phosphoglycerate). The peaks for phosphoenolpyruvate (PEP) and pyruvate were reached slightly later than the peak for phosphoglycerate.

Upon addition of NH_4^+, the levels of phosphoglycerate and of PEP declined

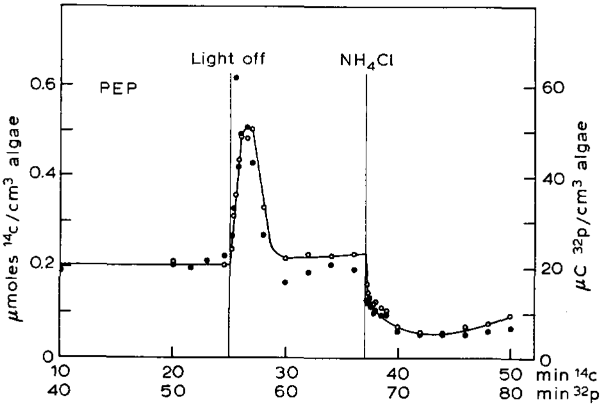

Fig. 2. Phosphoenolpyruvate. ○, ^{32}P label; ●, ^{14}C label.

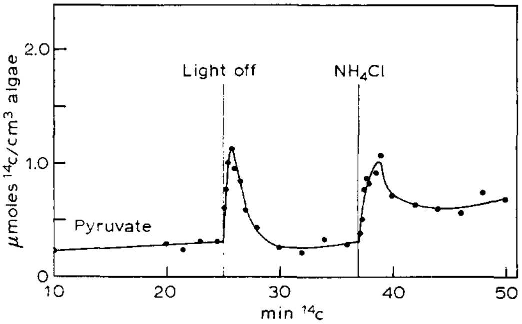

Fig. 3. Pyruvate.

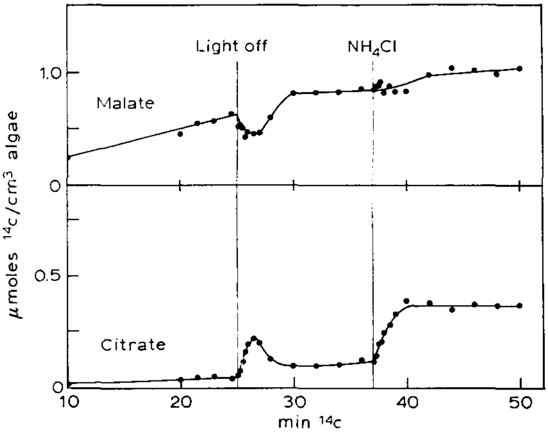

Fig. 4. Top: malate. Bottom: citrate.

rapidly to new, lower steady-state levels, while the level of pyruvate rose rapidly, passed through a peak, and then stabilized at a level more than twice as high as in the previous periods of photosynthesis and respiration without a nitrogen source in the medium.

When the light was turned off, the level of malate (Fig. 4) dipped for several minutes and then rose to a higher level than before. The level of citrate peaked during the first minutes, and then stabilized at a higher steady-state level than in the light. The level of malate was scarcely affected by the addition of NH_4^+ (in contrast to the result in the light, where it rose substantially[7]), while the level of citrate rose quickly to a much higher steady-state level.

With the light off, α-ketoglutarate rose to a higher level, glutamate rose and continued to rise, and glutamine was nearly unaffected (Fig. 5). After NH_4^+ addition, ketoglutarate level declined, but began to rise again towards the end of the experiment. The addition of NH_4^+ caused a momentary dip in the level of glutamate, followed by a sustained rise. Glutamine level rose rapidly.

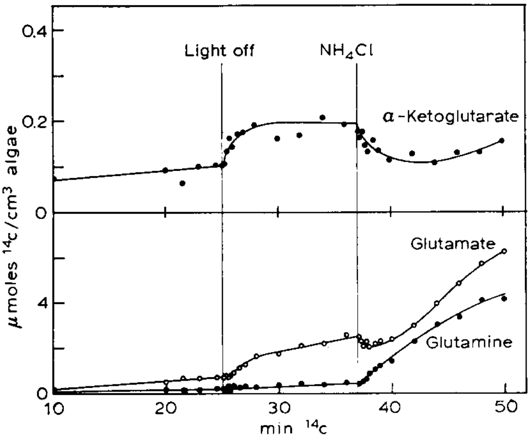

Fig. 5. Top: α-ketoglutarate. Bottom: ●, glutamine; ○, glutamic acid.

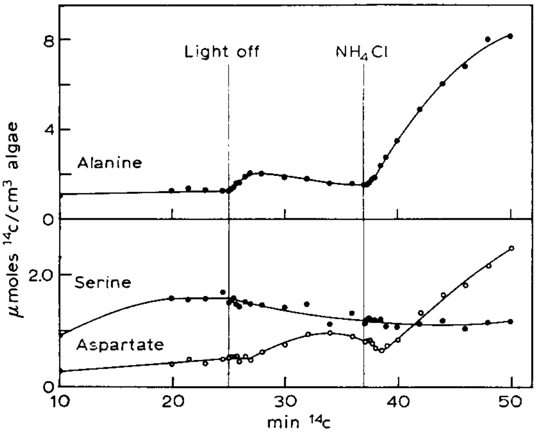

Fig. 6. Top: alanine. Bottom: ●, serine; ○, aspartate.

The levels of alanine and aspartate (Fig. 6) rose slightly with darkness; serine level declined slowly. With NH_4^+, the alanine pool increased very rapidly and immediately, that of aspartate first dipped and then rose rapidly.

As reported before[1], ATP level first dropped about 40 % when the light was turned off (Fig. 7) and then rose back to a higher steady-state level in the dark than in the light. Surprisingly, a very similar, though smaller transient dip occurred with the addition of NH_4^+. The changes in ADP level were reciprocal but smaller than in ATP labelling, even taking into account the ratio of phosphorus atoms per molecule.

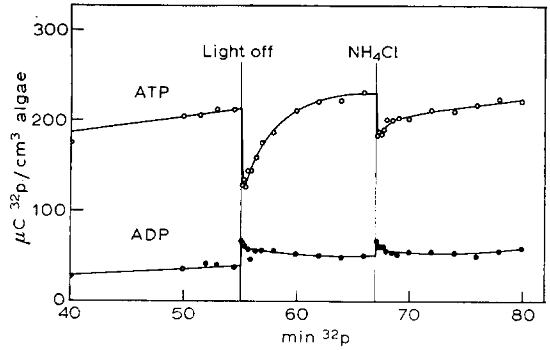

Fig. 7. Top: ATP. Bottom: ADP.

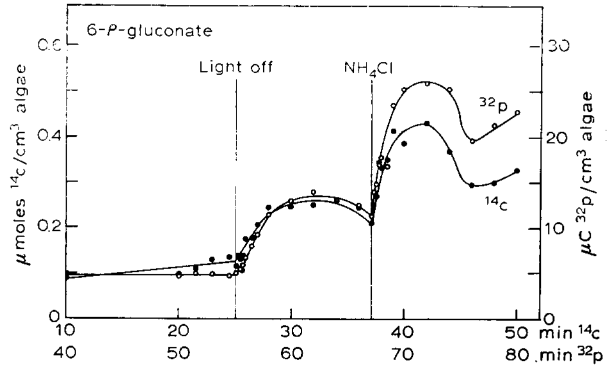

Fig. 8. 6-phosphogluconate ●, ^{14}C label; ○, ^{32}P label.

As found previously[1], the level of 6-phosphogluconate (Fig. 8) rises immediately upon darkening of the cells. An even more pronounced rise followed the addition of NH_4^+. The greater rise in ^{32}P label compared with ^{14}C in this and other compounds during the dark part of the experiment illustrates the respiration of endogenous starch and sugars which cannot be fully labeled with ^{14}C, even after 35 min photosynthesis with $^{14}CO_2$.

As noted earlier[1], the levels of sedoheptulose 1,7-diphosphate and fructose 1,6-diphosphate drop almost instantly when the light is turned off and then rise to new and higher levels in the dark (Fig. 9). With the addition of NH_4^+ the levels of both compounds first drop, and then rise again, declining again later in the experiment. The levels of pentose monophosphate and of dihydroxyacetone phosphate (Fig. 10) show similar but smaller effects.

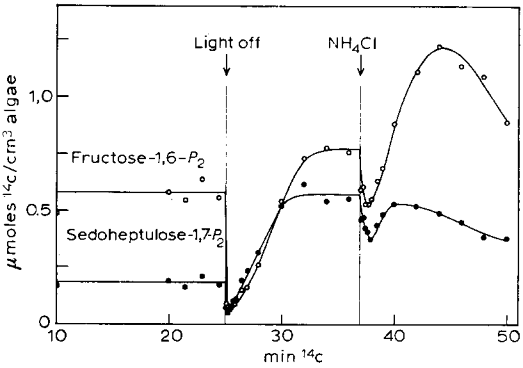

Fig. 9, ○, fructose 1,6-diphosphate; ●, sedoheptulose-1,7-diphosphate.

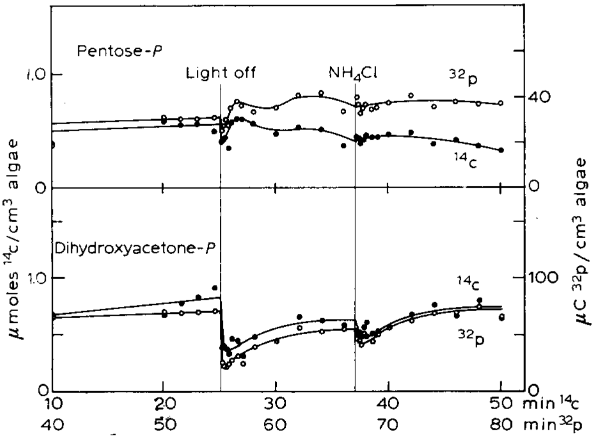

Fig. 10, Top: pentose monophosphate. Bottom: dihydroxyacetone phosphate. ○, ^{32}P label ●, ^{14}C label.

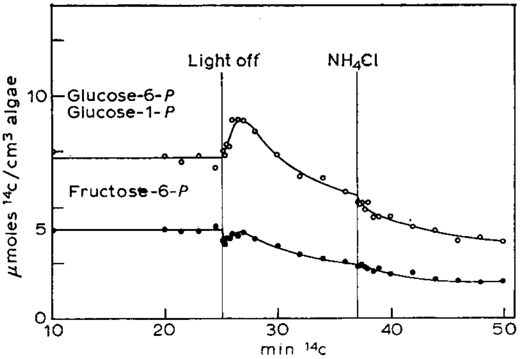

Fig. 11. ○, glucose 6-phosphate *plus* glucose 1-phosphate; ●, fructose 6-phosphate.

The level of glucose monophosphate (glucose 6-phosphate *plus* glucose 1-phosphate) peaks just after the light is turned off and then declines (Fig. 11). The level of fructose 6-phosphate first dips, then peaks (both very small) and then declines slowly. The ^{14}C label of both compounds continues to decline slowly after addition of NH_4^+.

When the light is turned off, starch accumulation ceases (Fig. 12), and the store of starch declines at a rate which remains constant for the duration of the experiment and is unaffected by the addition of NH_4^+. The rate of decrease in ^{14}C label in starch in the dark was 1.65 μmoles ^{14}C per min per cm^3 of algae, while the rate of decline of sucrose label after NH_4^+ addition was 0.85 μmole ^{14}C per min. Note that in this and in all data given in this paper we are following our previously adopted protocol[9] of expressing as μmoles ^{14}C the amount of ^{14}C found in μC divided by the specific activity of the administered $^{14}CO_2$ and $^{12}CO_2$ expressed as C per μmole of total carbon. The sucrose and especially the starch cannot be expected to be completely labeled ("saturated") after 25 min photosynthesis, so that the actual rate of disappearance of carbon from these carbohydrate reserves is presumably higher than the rates just reported.

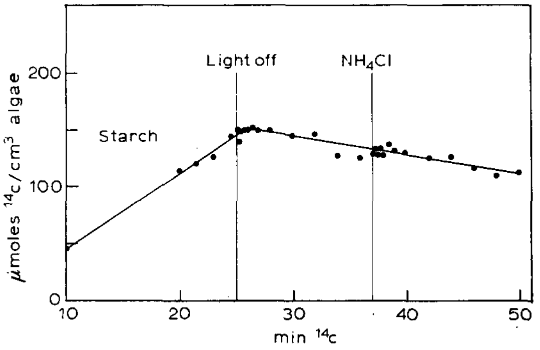

Fig. 12. Starch.

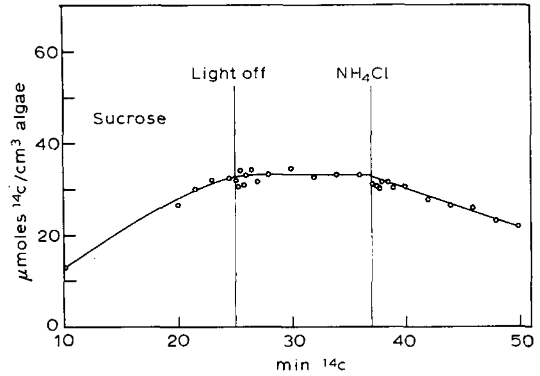

Fig. 13. Sucrose.

The level of ADPglucose (Fig. 14) declined very rapidly to below detectable levels, when the light was turned off. No ADPglucose was seen for the duration of the experiment. In contrast, the level of UDPglucose dipped slightly after the light was turned off, and again with the addition of NH_4^+ (Fig. 15).

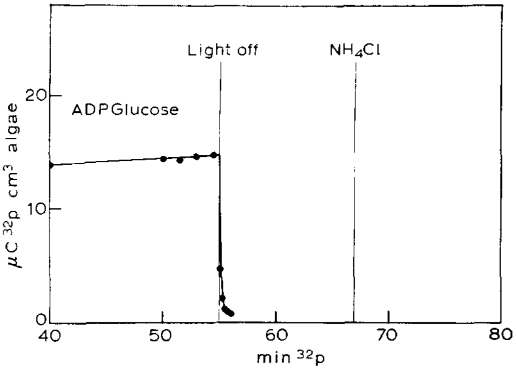

Fig. 14. ADPglucose.

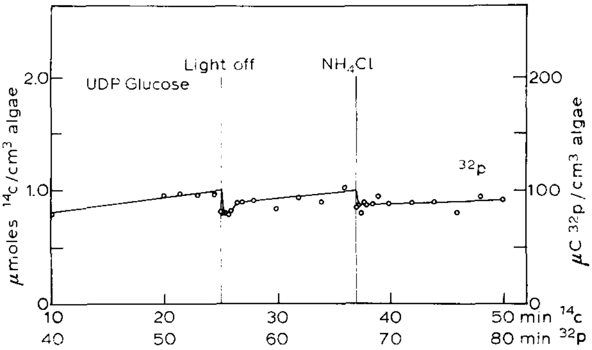

Fig. 15. UDPglucose.

DISCUSSION

Starch and sucrose metabolism

The general pattern of carbon flow and its regulation emerging from these data is similar to that established previously[1,2,11], but the present information is more complete. It is now clear that in the absence of added nitrogen source in the medium, much of the carbon for respiration is derived from starch as soon as the cells are darkened. That this utilization of starch was completely unaffected by the addition of NH_4^+ while the breakdown of sucrose was stimulated by NH_4^+ and was unaffected by darkening is further evidence for the proposal that the regulatory sites for the metabolism of starch and of sucrose are quite different. In the light addition of NH_4^+ completely stopped the increase in sucrose, but only decreased the rate of starch formation about 40 %. This led to the suggestion that the addition of NH_4^+ to the medium in some way causes inhibition of sucrose phosphate synthetase (EC 2.4.1.14).

However, the present experiment indicates a stimulation in the breakdown of sucrose upon NH_4^+ addition. It may be that the breakdown of sucrose proceeds *via* a different path and at a different site than its synthesis. The most likely path for sucrose breakdown would seem to be the reaction mediated by UDPglucose: D-fructose 2-glucosyltransferase (EC 2.4.1.3) in which sucrose and UDP are converted to UDPglucose and fructose. Presumably, this reaction might occur in the cytoplasm and would be stimulated upon the addition of NH_4^+ to the cell suspension in the dark. According to a recent report[12] the isolated enzyme from *Phaseolus aureus* is inhibited by NADPH. Other data to be discussed in the present study (Fig. 8) strongly suggest that both darkness and NH_4^+ addition cause a decrease of the level of NADPH in *Chlorella pyrenoidosa.*

The simplest interpretation for the small dips in the otherwise constant level of UDPglucose occurring when the light is turned off and when NH_4^+ is added is that the level of UDPglucose is reflecting the level of ATP (Fig. 7) with the reactions converting ATP and UDP to ADP and UTP, and glucose 1-phosphate and UTP to UDPglucose and pyrophosphate being rapidly reversible under all conditions of the experiment.

The sudden drop in the level of ADPglucose to below detectable limits (Fig. 14) and the fact that it is not seen at any time later in the dark despite the high steady-state levels of ATP and glucose monophosphates provides strong support for the importance *in vivo* of the regulatory role in starch synthesis of the enzyme ATP: D-glucose 1-phosphate uridylyltransferase (EC 2.7.7.-) which mediates the reaction of glucose 1-phosphate with ATP to give ADPglucose and pyrophosphate. Studies of the properties of the isolated enzyme from leaf chloroplasts[13–16] showed activation by phosphoglycerate and fructose 6-phosphate and inhibition by inorganic phosphate. However, in the present study, as in some previous studies[1,2], the level of neither phosphoglycerate nor fructose 6-phosphate appear to change enough to effect the change in enzymic activity suggested by the *in vivo* drop in ADPglucose activity. The level of inorganic phosphate inside the chloroplasts is unfortunately difficult to determine. It could be one of the factors regulating the activity of this enzyme *in vivo.*

Oxidative pentose phosphate cycle

The sudden large rise in the level of 6-phosphogluconate (Fig. 8) when the light is turned off has been explained as resulting from activation of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) by a decrease in the NADPH/NADP+ ratio which occurs when photoelectron transport from water to NADP+ stops[3,4]. The appearance of 6-phosphogluconate is taken to be an indication of the operation of the oxidative pentose phosphate cycle. Even with the light on, the oxidative pentose phosphate cycle can be made to operate in algae or in isolated spinach chloroplasts by the addition of vitamin K_5, which, in its oxidized form, diverts electrons from the reduction of NADP+ (refs. 3, 4). The inhibition of glucose 6-phosphate dehydrogenase by high ratios of NADPH/NADP+ or by NADPH, or its activation by NADP+, seems likely to be by means of a conformational change in the enzyme leading to a large change in catalytic activity, since the physiological standard free energy change, $\Delta G'$, for the conversion of glucose 6-phosphate, NADP+, and water to 6-phosphogluconate is about -8 kcal[17].

With the addition of NH_4^+ in the dark, the rise in 6-phosphogluconate (Fig. 8) was even more pronounced than when the light was turned off. There could be other ways in which NH_4^+ addition stimulates glucose 6-phosphate dehydrogenase and, hence, the oxidative pentose phosphate cycle, but we interpret this effect as an indication that the $NADPH/NADP^+$ has tended to drop still more upon the addition of NH_4^+. The mechanism for this effect appears to be a partial uncoupling of electron flow associated with oxidative phosphorylation. The level of ATP dropped sharply and remained somewhat lower when NH_4^+ was added in the dark (Fig. 7). The partial recovery in ATP level after several minutes may be expected due to the increased flow of carbon into the tricarboxylic acid cycle resulting from the stimulation of pyruvate kinase (EC 2.7.1.40) as discussed below. The increased flow of carbon in the tricarboxylic acid cycle leads, in turn, to more oxidative electron transport and more ATP even though the coupling is poorer.

Although NH_4^+ ions are known to uncouple photophosphorylation in broken chloroplasts[18], our previous study[7] showed no decrease in ATP level when 1 mM NH_4^+ was added to photosynthesizing *Chlorella pyrenoidosa*. However, the drop in ATP level with NH_4^+ addition in the dark (Fig. 8), together with the evidence for increased flow of carbon through the tricarboxylic acid cycle, shows clearly the uncoupling effect for oxidative phosphorylation.

The drop in levels of fructose 1,6-diphosphate and sedoheptulose 1,7-diphosphate (Fig. 9) and in dihydroxyacetone phosphate (Fig. 10) when the light is turned off is due to the sudden lowering of the levels of ATP and NADPH when the photoelectron transport stops, leading to a reversal of the reactions mediated by triose phosphate dehydrogenase (EC 1.2.1.13) and phosphoglycerate kinase (EC 2.7.2.3) so that glyceraldehyde-3-phosphate is oxidized to 3-phosphoglycerate. Operation of the oxidative pentose phosphate cycle supplies new carbon to the fructose diphosphate–sedoheptulose diphosphate–triose phosphate pool, partially restores the levels of NADPH and ATP, and thus leads to a rise in the levels of these sugar phosphates after several minutes.

Very nearly the same sequence of events occurs upon the addition of NH_4^+, except that now the transient drop in ATP and NADPH is due to uncoupling of oxidative phosphorylation by NH_4^+ (if our interpretation above is correct). Consequently, the levels of the two sugar diphosphates and of dihydroxyacetone phosphate again drop momentarily and then rise again. In this case, the restoration of levels of ATP and NADPH due to increased electron transport is also aided by the stimulation of pyruvate kinase, leading to more carbon flowing into the tricarboxylic acid cycle.

Intracellular localization of metabolism and control

Since starch synthesis and storage occurs inside the chloroplasts, the breakdown of starch and metabolism of glucose 6-phosphate *via* the oxidative pentose phosphate cycle must occur in the chloroplasts. The product of that cycle, triose phosphate as well as 3-phosphoglycerate, can either diffuse or be translocated out of the chloroplasts, and probably represents a major export from chloroplasts in either light or dark. The site of sucrose synthesis may be in the chloroplasts, but the catabolism of sucrose could well be in the cytoplasm, though there seems to be no clear evidence on this point. Once in the cytoplasm, phosphoglycerate gives rise to phosphoenolpyruvate and pyruvate, which then may enter the metabolism of the mitochondria.

The regulatory effects discussed in this paper seem to suggest that the levels of ATP and NADPH in the dark, which should be controlled by oxidative electron transport in the mitochondria, exert strong regulatory effects on the oxidative pentose phosphate cycle and the oxidation of triose phosphate, and perhaps on sucrose breakdown as well. Perhaps both sucrose breakdown and oxidative pentose phosphate cycle can be located in the cytoplasm and regulated by the ATP and NADPH levels controlled from the mitochondria.

Phosphoglycerate to pyruvate

The sudden rise in 3-phosphoglycerate level when the light is turned off is due to the reversal of the reduction of phosphoglycerate to triose phosphate, as discussed earlier, stemming from the sudden lowering of the levels of ATP and NADPH. By the time these levels are partially restored, the carboxylation reaction which forms phosphoglycerate during photosynthesis has stopped[1,2,4]. Then the flow of carbon to pyruvate and biosynthesis causes the level of phosphoglycerate to fall until its formation by triose phosphate oxidation is balanced by its utilization.

3-Phosphoglycerate, 2-phosphoglycerate and PEP are rapidly, though not instantly, interconvertible. The two phosphoglycerates are not separated by the chromatography used. About 10 % of the total would be 2-phosphoglycerate at equilibrium, while the ratio of phosphoglycerate to PEP would be about 2.5 at equilibrium. Under the steady-state light conditions in this experiment, the ratio of phosphoglycerate:PEP is about 16, and drops to about 10 in the subsequent dark periods.

The transient peak in PEP is relatively much higher and persists considerably longer than the peak in phosphoglycerate when the light is turned off. This suggests that there might be another source in addition to phosphoglycerate for the formation of PEP during this transient period. One possibility is the decarboxylation of oxaloacetate, but it is not clear what the stimulus for such a decarboxylation would be.

The rapid rise and peak in pyruvate level when the light is turned off (Fig. 3) probably reflects both the peak in PEP and the 40 % dip in level of ATP, together with the small peak in ADP (Fig. 7).

$$\mathrm{PEP}^{3-} + \mathrm{ADP}^{3-} + \mathrm{H}^{+} \rightarrow \mathrm{pyruvate}^{-} + \mathrm{ATP}^{4-}$$

Calculation of free energy changes (ΔG^{s}) accompanying reactions of steady state in photosynthesizing *C. pyrenoidosa* (rates of energy dissipation as heat accompanying reactions) showed that steps with large negative values of ΔG^{s} were usually steps known to be metabolically regulated[17]. The physiological standard free energy change for the pyruvate kinase reaction in the direction of PEP hydrolysis (pH 7) was calculated to be $\Delta G' = -5.6$ kcal. From the data of McQUATE AND UTTER[19], the logarithm of the equilibrium constant (not including H^+) at pH 7.7 is calculated to be 4.28. In the present study, the ratio [pyruvate][ATP]/[PEP][ADP] is estimated to be 3.8 both in light and in the dark (after the transient) before $\mathrm{NH_4}^+$ addition. After the $\mathrm{NH_4}^+$ addition, the ratio is estimated to be 30. (In this estimate, the $^{32}\mathrm{P}$ labelling of ATP is divided by 2 for comparison with ADP on the assumption that the phosphate group closest to the ribose moiety is only slightly labelled, but allowance is made for the slow labelling of that phosphate group indicated by the slope of the

ATP curve during steady-state periods.) Thus, $\Delta G^{s}_{7.7} = -1.363\ (4.28-\log 3.8) = -5.04$ kcal before NH_4^+ addition, and $\Delta G^{s}_{7.7} = -3.82$ kcal after NH_4^+ addition. Corresponding ratios of forward to back reactions are 5020 before NH_4^+ addition and 630 after the addition. These are comparable to the changes observed in our previous study when NH_4^+ was added in the light to photosynthesizing *C. pyrenoidosa*, $\Delta G^{s}_{7.7}$ being -4.47 kcal before addition and -3.65 kcal after addition in that study.

It may be concluded that the stimulation of the pyruvate kinase reaction by the addition of 1 mM NH_4^+ depends very little, if at all, on whether the algae are photosynthesizing in the light or respiring in the dark.

The tricarboxylic acid cycle and amino acid biosynthesis

Alanine formation appears to depend critically on pyruvate concentration. When the light was turned off, the transient peak in pyruvate level led to an increase in alanine, which stopped once the pyruvate concentration returned to its former level. With the addition of NH_4^+ and consequent rise in pyruvate, alanine concentration increases very rapidly (Fig. 6).

Since the carbon skeleton for serine comes from phosphoglycerate, there is no increase in the level of serine (Fig. 6) either with dark or with NH_4^+ addition. This result shows that the amino acid synthesis, under conditions of this experiment, depends very much on the supply of carbon skeletons (α-keto acids) but is insensitive to the level of glutamate over the range existing in this experiment.

Early studies of carbon metabolism in green cells showed that the level of glutamate rises rapidly when the light is turned off[11], even without the addition of NH_4^+. It was proposed that darkening of the cells leads to an increased rate of flow of carbon from the carbohydrate and sugar phosphate pools into the biosynthetic metabolism. The present study shows that although there is a surge of carbon through the pyruvate kinase reaction during the first 3 min after the light is turned off, the conversion of PEP to pyruvate then proceeds at about the same rate as during steady-state conditions in the light, as discussed in the last section. Nevertheless, there is an accelerated flow of carbon into citrate, ketoglutarate and glutamate. Probably this is because lipid synthesis, using acetyl CoA, has greatly slowed or stopped, due to the lower levels of NADPH in the dark. Thus, more acetyl CoA becomes available for condensation with oxalacetate to make citrate.

With the addition of NH_4^+, there is an accelerated flow of carbon to pyruvate and to acetyl CoA, and a large increase in the steady-state level of citrate. More carbon must flow into the α-ketoglutarate pool, but the level drops due to the accelerated reductive amination of this ketoacid to make glutamate. This reductive amination and subsequent amidation lead to rapidly increasing levels of glutamate and glutamine. However, the increased rate of transamination reactions (particularly of pyruvate) causes the glutamate pool to dip when the NH_4^+ is first added.

Although malate level increased upon NH_4^+ addition in the light[7], it does not increase with NH_4^+ addition in the dark (Fig. 4). Probably this is due to the lower ratio of $NADPH/NADP^+$ in the dark compared to light.

GENERAL CONCLUSIONS

With *Chlorella pyrenoidosa*, which have been photosynthesizing without a nitrogen source in the medium, the transition to dark metabolism is triggered by a sudden drop in the levels of NADPH and ATP. There is an immediate cessation of starch synthesis, a stimulation of starch breakdown, activation of the oxidative pentose phosphate cycle by activation of glucose 6-phosphate dehydrogenase (and inactivation of the carboxylation, hexose diphosphatase, and phosphoribulokinase reactions of the reductive pentose phosphate cycle), a transient but not a permanent increase in the activity of pyruvate kinase, cessation or decrease in fatty acid synthesis, an increased flow of carbon into the tricarboxylic acid cycle due to the greater availability of acetyl CoA no longer used for fatty acid synthesis, consequent greater electron flow and oxidative phosphorylation restoring the level of ATP following the transient dip when the light was turned off, and a consequent greater rate of formation of glutamate.

Upon the addition of NH_4^+, primary effects appear to be stimulation of the rate of conversion of PEP to pyruvate (mediated by pyruvate kinase) and partial uncoupling of oxidative phosphorylation, leading to lowered levels of NADPH and ATP. There is stimulation of sucrose breakdown, further activation of glucose 6-phosphate dehydrogenase and the oxidative pentose phosphate cycle, more carbon flow into the tricarboxylic acid cycle, consequent greater electron flow partially restoring the ATP level, and accelerated reductive amination of α-ketoglutarate and increased amino acid synthesis.

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